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Simultaneous determination of purine bases, ribonucleosides and ribonucleotides by capillary electrophoresis–electrochemistry with a copper electrode

Hua Lin, Dan-Ke Xu, Hong-Yuan Chen*

Department of Chemistry, Nanjing University, Nanjing 210093, China

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Abstract

Simultaneous determination of purine bases, ribonucleosides and ribonucleotides was achieved by coupling capillary electrophoresis (CE) with wall-jet amperometric detection. A 200 μm diameter copper disk electrode was applied at working potential, +0.65 V vs. saturated calomel electrode. The current response of high sensitivity and stability was obtained in strong basic solutions which were suitable for satisfactory CE separations. The calibration curve was linear over 2–3 orders of magnitude and the limits of detection for adenine, guanine, xanthine, uric acid, adenosine, guanosine, adenosine-5′-monophosphate and guanosine-5′-monophosphate were below 9 fmol ($S/N=3$). The use of this method for the separation and detection of compounds present in human plasma samples was reported.

Keywords: Detection, electrophoresis; Electrodes; Purines; Ribonucleosides; Ribonucleotides

1. Introduction

The separation and detection of purine compounds is an interesting and challenging topic because these compounds are involved in a large number of biochemical processes. In the past, several analytical methods based on high-performance liquid chromatography (HPLC) have been developed for the determination of nucleobases, nucleosides and nucleotides [1–8] followed by spectrophotometric and radioisotopic detection. However, some of these methods have low column efficiencies and long analysis times.

Capillary electrophoresis, where resolution occurs due to difference in the mobilities of ions in an electric field, has been proved to have several advantages over HPLC, particularly in terms of its high efficiency and small volume requirements [9]. This makes it possible to analyse a number of sophisticated biological and clinical samples, even if amounts are limited. Nevertheless, the small column dimensions and extremely small sample zone widths prevent detection coupling with this method.

Electrochemical detection (ED) in liquid-phase separations has shown to be a powerful method for the determination of a wide range of analytes due to the fact that it can usually be performed without loss in sensitivity and selectivity. Utilizing amperometric detection, several purine compounds have been detected following the separation by HPLC using a ordinary carbon electrode [10–12]. Unfortunately, electrooxidation of these compounds requires a

*Corresponding author.

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relatively high oxidation potential (+1.1 V vs. Ag/AgCl), which limits the selectivity. However, a copper electrode, in combination with strongly alkaline condition, could offer a promising way to minimize overpotential effects because the detection is based on a electrocatalytic oxidation mechanism [13,14] and not on the direct electrooxidation of the compound of interest. The applications of the copper electrode have been reported for the detection of carbohydrate compounds [15], amino acids and peptides [16] by CE–ED. To the best of our knowledge, the use of a copper electrode for simultaneous detection of a mixture of ribonucleotides, ribonucleosides and bases in a single CE run has not been investigated in microcolumn separations.

In this paper, we will present the separation and detection of several biologically important purine-containing compounds by CE–ED. In this case, a significant advance for the determination of purines is use of copper as electrode material. This greatly increases the stability of the electrode and allows low limits of detection. The sensitivity and selectivity of the method is demonstrated by the detection of some purine compounds in human plasma.

2. Experimental

2.1. Reagents

The purine standards were purchased from Sigma (St. Louis, MO, USA) without further purification. All other chemicals were analytical reagent grade, and all chemicals were used as received. All solutions were prepared with doubly distilled water and stored at 4°C. The required sample solutions were obtained by serial dilution in the separation electrolyte.

2.2. Apparatus

Electrophoresis in the capillary was driven by a 30 kV high-voltage power supply (Shanghai Third Analytical Instrument Factory, Shanghai, China). An uncoated fused-silica capillary (38 cm×25 μm I.D.) was obtained from Yongnian Optical Fiber Factory (Hebei, China). Sample introduction was accomplished electrokinetically and the volume injected was calculated in the continuous fill mode by recording the time required for the sample to reach the detector. In addition, the outlet end of the capillary was always maintained at ground. The electrochemical cell was shielded in a copper box to reduce external disturbance.

The working electrode was constructed with a 200 μm diameter Cu wire. A glass capillary tube was pulled with a vertical pipet puller (Shanghai Institute of Physiology, Shanghai, China) to a narrow tip, which was cut to allow the passage of the Cu wire. The wire was then inserted through the capillary until it protruded approximately 3–4 mm from the tip. At the junction of the capillary and the Cu wire, epoxy glue was applied to the tip to seal the Cu wire to it. The other end of the wire was also epoxied to support the electrical connection. Once cured, the Cu wire was cut with surgical scissors. Before each utilization, the electrode surface was wet polished with 0.05 μm alumina powder, rinsed with a stream of deionized water and sonicated for a few minutes.

Constant-potential amperometric detection with CE was performed using the wall-jet approach [17]. A conventional three-electrode mode was used with the Cu working electrode at the end of the separation capillary, and the electrodes were connected to an amperometric detector (Shanghai Institute of Organic Chemistry, Academy of Sciences of China, Shanghai, China), which provided potential control and current output. The electropherograms were monitored with a strip-chart recorder (DaHua Electric Instrument and Meter Plant, Shanghai, China). The detector potential was fixed at +0.65 V vs. saturated calomel electrode (SCE) unless otherwise indicated.

In order to make the separation reproducible, the NaOH electrolyte solution was freshly prepared and replaced before each run to avoid the decrease in separation current resulting from the presence of low concentrations of carbon dioxide [15,16].

2.3. Cyclic voltammetry

All cyclic voltammetry (CV) experiments were carried out with a computerized electrochemical analyzer (BAS100B, USA). A three-electrode cell was used, which consisted of a copper working electrode, a saturated calomel electrode and a platinum auxiliary electrode. All cyclic voltammo-

grams were obtained in 40 m*M* NaOH using a scan rate of 50 mV/s.

2.4. Sample preparation

100 μl of human plasma obtained from a volunteer was extracted with an equal amount of trichloroacetic acid (10%, v/v). The extract was centrifuged for 5 min at 3000 *g*. The supernatant was neutralized with 0.1 *M* NaOH. This solution was diluted immediately with 200 μl running buffer and directly injected onto the CE system.

3. Results and discussion

3.1. Electrochemistry

The initial study involved the CV behavior of the purine compounds. Fig. 1 depicts the CV of adenine, adenosine and 5′-AMP in 40 m*M* NaOH solution at the Cu electrode. Interestingly, the behavior seen was similar to that exhibited by both carbohydrates [15,18,19] and amino acids, peptides and proteins [13,14,19]. In particular, these biomolecules gave a continuous increase in anodic current between +0.30 V and +0.70 V vs. SCE. As can be seen the CV behavior for these compounds at higher potentials was not uniform in both the amount of oxidation current and the exact potential at which it occurred. For some compounds, such as adenine and adenosine, the response of anodic current showed a much larger enhancement whereas, for 5′-AMP, the current levels observed increased slightly and the oxidation wave was very similar to voltammetric background. The partial loss of the electrode response was probably attributable to the modification of the adenosine with phosphate. On the other hand, there were subtle differences in the anodic peak potentials of the different purine compounds. Beyond this, the amperometric response showed a slight increase upon the increasing of the NaOH concentration from 20 m*M* to 50 m*M*.

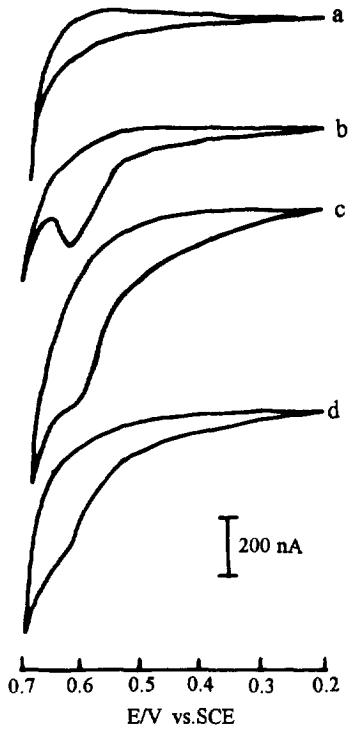

Fig. 1. Cyclic voltammograms at a copper electrode in 0.04 *M* NaOH. Analyte: (a) blank, (b) Ade, (c) Ado, (d) 5′-AMP; all concentrations, $5 \cdot 10^{-4}$ *M*; scan rate, 50 mV/s.

Finally, given the peak potential wave corresponding to the Cu(II)/Cu(III) redox couple, the detection processes involved are most likely attributed to the Cu(III) species participating in the catalytic oxidation of the analytes. While the detailed mechanism for the oxidation of purine-containing compounds at the Cu electrode needs further investigation, these electrochemistry results, however, do clearly indicate the most immediate and attractive possibilities of constant potential amperometric detection of purine bases and related compounds for capillary electrophoresis.

3.2. CE–ED of purine compounds using a Cu disk electrode

To assess the effect of the flowing conditions on the anodic current response as a function of the applied potential, hydrodynamic voltammograms are plotted in Fig. 2 for several purines. The experiments were performed under CE conditions with wall-jet amperometric detection. After each change in applied potential, 5–10 min of stabilization time was allowed prior to injection of the sample. The different compounds showed almost the same trend in

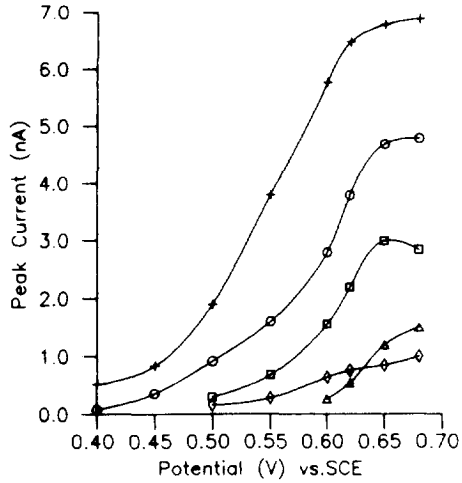

Fig. 2. Hydrodynamic voltammograms of (+) Gua, (○) Ado, (□) Guo, (△) Ade and (◇) 5′-AMP (concentrations between 20 and 120 μ*M*) at a copper electrode. Separation voltage, 12.5 kV; injection by electromigration, 3 s at 12.5 kV; buffer, 40 m*M* NaOH.

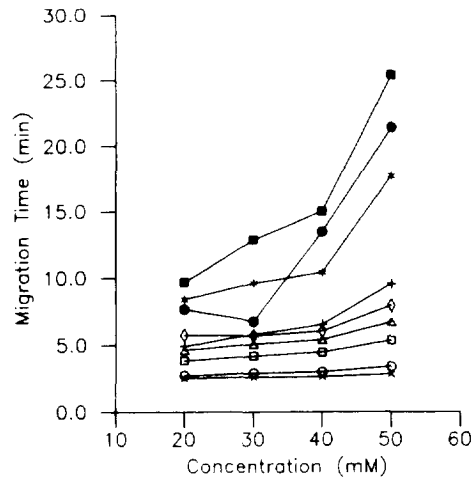

Fig. 3. Effect of concentration of NaOH on separation. Detection potential, +0.65 V vs. SCE; other conditions as in Fig. 2. ○=Ado; □=Guo; △=Ade; ◇=5′-AMP; +=Gua; *=5′-GMP; ●=Xan; ■=UA; ×=neutral marker.

response except that the amplitudes were different, which was consistent with their CV behaviours. Furthermore, the current signals almost reached their maximum levels at approximately +0.65 V for purine bases and related compounds examined. Above this potential, there was a significant increase in the background current due to the onset of oxygen evolution. According to the hydrodynamic voltammograms, an operating potential of +0.65 V vs. SCE gave the best compromise between signal and background, and therefore was selected for subsequent CE amperometric detection in alkaline solution.

In an attempt to optimize the separation of purine compounds, the composition of the electrophoresis medium was changed with 10, 20, 30, 40 and 50 m*M* sodium hydroxide. These basic conditions also satisfied the requirements for the sensitive detection of these samples at the copper electrode. The relationship between the migration time of each compound and the concentration of sodium hydroxide is shown in Fig. 3. As the hydroxide ion concentration was increased, the purines examined became more negative due to dissociation. The migration time, accordingly, increased with slight differences in the migration order, and the resolution improved without increasing the current intensity too much. When the buffer concentration was further increased above 40 m*M*, the migration time became too long, and the separation did not improve. Thus, the optimum concentration was fixed at 40 m*M*, giving short migration time and a good separation quality.

The influence of the applied voltage on separation characteristics was also investigated. An increase of voltage from 10 kV to 18 kV neither enhanced the resolution of the analytes, nor changed the elution order. However, it did only reduce the migration time. In view of the Joule heating problem and the generation of high background, 12.5 kV was chosen as the separation voltage.

The detector stability under CE–ED conditions is shown in Fig. 4. The working potential (+0.65 V vs. SCE) had been applied to the same Cu electrode with the surface being renewed for about 30 min before the first sample injection. The results shown represented the current response for 20 injections of 120 μ*M* adenine and 80 μ*M* adenosine solution over a period of approximately 5 h in 40 m*M* sodium hydroxide. It was observed that the activity of fresh copper electrodes decreased slightly within the first two injections. After the subsequent injections, the peak responses tended to decrease quite slowly and gave R.S.D. values of 6.5% and 5.7%, respectively,

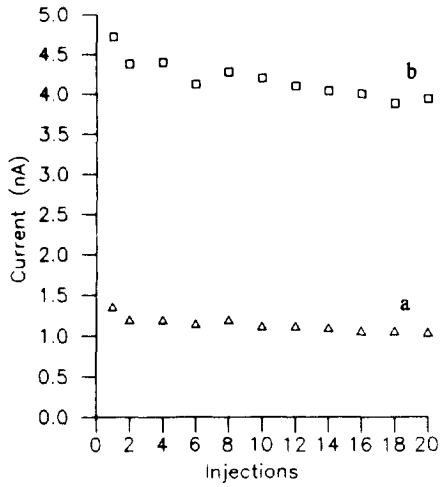

Fig. 4. CE–EC peak current change for (a) 120 μ*M* Ade and (b) 80 μ*M* Ado; electrode potential, +0.65 V vs. SCE; injection of the sample every 15 min. Other conditions are similar to those in Fig. 2.

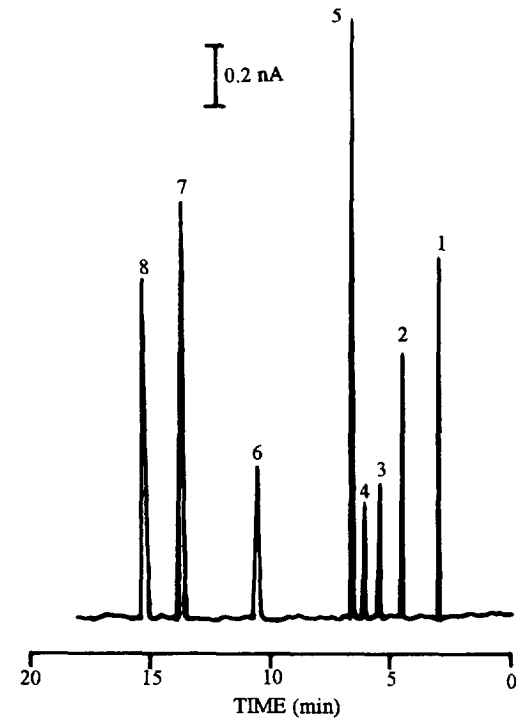

Fig. 5. Electropherogram of (1) 20 μ*M* Ado; (2) 20 μ*M* Guo; (3) 40 μ*M* Ade; (4) 20 μ*M* 5′-AMP; (5) 10 μ*M* Gua; (6) 20 μ*M* 5′-GMP; (7) 20 μ*M* Xan; (8) 20 μ*M* UA. Other conditions as in Fig. 4.

which was in agreement with previous reports for amino acids and peptides [16]. The stabilization of the baseline under the very basic conditions was another parameter of practical applications. A stable background current was obtained after the electrode had been subjected to the working potential for approximately 30 min. As far as CE–ED applications were concerned, the same copper electrode was used without any further treatment at least 2–3 days.

Finally, as shown in Fig. 5, CE–ED using the copper electrode in 40 m*M* NaOH proved to be very effective in separation and detection of a sample mixture of ribonucleotides, ribonucleosides and bases. The electropherogram demonstrated a good resolution and a stable baseline. The linearity of response was evaluated over a wide range of concentrations above the detection limits, and usually extended over 2–3 orders of magnitude with correlation coefficients (r) of at least 0.99. The limits of detection were calculated to be below 9 fmol at a S/N of 3 for the 8 compounds examined. In brief, the results of these experiments are summarized in Table 1.

3.3. Analytical application

The application of the CE–ED system with a copper electrode has been performed to detect several purine compounds in human plasma according to the analytical conditions described above. Fig. 6A illustrates an electropherogram of a trichloroacetic acid extract obtained from a healthy human volunteer. The system allowed the separation and simultaneous detection of five purine compounds in plasma samples. The electropherogram of a standard mixture solution of these five compounds, for comparison, is shown in Fig. 6B to match the migration times. However, the migration time of some purines in the biological sample was observed to be different from that in the standard solution. The matrix effect might account for this phenomenon [20]. The presence of these purine compounds in the plasma sample was further confirmed by means of spiking each standard solution into the extract sample. This result was comparable to those of other systems using UV detection [21]. The determination of the other purines in human plasma has not yet been

Table 1
CE–ED of purine compounds at the Cu electrode

Peak[a]	Compound	Linear range (M)	r^{b}	Detection limit[c] (fmol)
1	Adenosine (Ado)	$1\cdot10^{-6}$–$2\cdot10^{-3}$	0.997	1.8
2	Guanosine (Guo)	$1\cdot10^{-6}$–$2\cdot10^{-3}$	0.995	1.8
3	Adenine (Ade)	$5\cdot10^{-6}$–$5\cdot10^{-4}$	0.999	9.0
4	Adenosine-5′-monophosphate (5′-AMP)	$5\cdot10^{-6}$–$5\cdot10^{-4}$	0.999	9.0
5	Guanine (Gua)	$4\cdot10^{-7}$–$5\cdot10^{-4}$	0.995	0.7
6	Guanosine-5′-monophosphate (5′-GMP)	$2\cdot10^{-6}$–$5\cdot10^{-4}$	0.992	3.6
7	Xanthine	$5\cdot10^{-7}$–$5\cdot10^{-4}$	0.990	0.9
8	Uric acid	$1\cdot10^{-6}$–$1\cdot10^{-3}$	0.997	1.8

Experimental conditions: amperometric detection at +0.65 V vs. SCE in 0.040 M NaOH; electrophoresis conditions as in Fig. 5.
[a] Peak numbers were the same as those used for peak referencing in Fig. 5.
[b] The number of points for the linear regression calculation was at least five.
[c] The injection volume was estimated to be 1.8 nl.

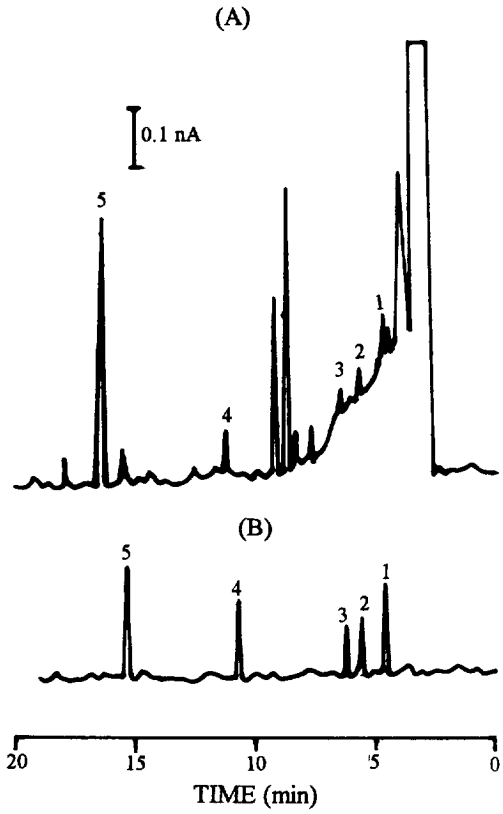

Fig. 6. Electropherogram of (A) human plasma sample and (B) purine standards. (1) Guo; (2) Ade; (3) 5′-AMP; (4) 5′-GMP; (5) UA. Experimental conditions are the same as those described in Fig. 4.

achieved because of the limits of detection being relatively higher and the sample pretreatment procedure. The sensitivity of this method may be the subject of further improvement, and the application to other biological samples is envisaged.

4. Conclusions

The wall-jet ED system with a copper electrode has been evaluated to be suitable for the simultaneous determination of purine bases, ribonucleosides and ribonucleotides by CE. The copper electrode is easy to prepare and has excellent properties including stability and sensitivity. The method has been proved to have a wide linear response range and low detection limits. In addition, the conditions employed are suitable for the efficient separation and amperometric detection of these purine-containing compounds without any divergence. An application of this method to the analysis of these compounds in a biological sample is performed with a simple and sensitive approach under basic conditions. It is believed that this method could be applicable to many other interesting purine compounds.

Acknowledgments

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